Reference: *Biol. Bull.* **198:** 319–331. (June 2000)

Catecholamines Modulate Metamorphosis in the Opisthobranch Gastropod *Phestilla sibogae*

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Abstract. Larvae of the nudibranch *Phestilla sibogae* are induced to metamorphose by a factor from their adult prey, the coral *Porites compressa.* Levels of endogenous catecholamines increase 6 to 9 days after fertilization, when larvae become competent for metamorphosis. Six- to nine-day larvae, treated with the catecholamine precursor L-DOPA (0.01 m*M* for 0.5 h), were assayed for metamorphosis in response to coral inducer and for catecholamine content by high-performance liquid chromatography. L-DOPA treatment caused 20- to 50-fold increases in dopamine, with proportionally greater increases in younger larvae, so that L-DOPA-treated larvae of all ages contained similar levels of dopamine. A much smaller (about twofold) increase in norepinephrine occurred in all larvae. The treatment significantly potentiated the frequency of metamorphosis of 7- to 9-d larvae at low concentrations of inducer. In addition, L-DOPA treatment at 9 d increased aldehyde-induced fluorescence in cells that were also labeled in the controls, and revealed additional cells. However, all labeled cells were consistent with the locations of cells showing tyrosine-hydroxylase-like immunoreactivity. Catecholamines are likely to modulate metamorphosis in *P. sibogae,* but rising levels of catecholamines around the time of competence are insufficient alone to account for sensitivity to inducer in competent larvae.

Received 6 August 1999; accepted 20 January 2000.

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Abbreviations: α-MMT, α-methyl-DL-*m*-tyrosine; CI, coral inducer; DA, dopamine; DHBA, dihydroxybenzylamine; DOPAC, dihydroxyphenylacetic acid; FaGlu, formaldehyde-glutaraldehyde; HPLC, high performance liquid chromatography; L-DOPA, L-3,4-dihydroxyphenylalanine; MBL-ASW, MBL artificial seawater; NE, norepinephrine; PBS, phosphate-buffered saline; TH-LIR, tyrosine-hydroxylase-like immunoreactive.

Introduction

The larvae of many marine invertebrates settle and metamorphose in response to chemical signals in the environment. In some taxa, larvae become competent to respond to such chemical inducers only after an obligate planktonic phase (Pechenik, 1990; Hadfield and Strathmann, 1996). In gastropod molluscs, the minimum time to competence ranges from several days to several weeks and may or may not require feeding and growth, depending on the species. For example, the onset of competence occurs in a predictable, age-dependent fashion in the lecithotrophic larvae of the opisthobranch *Phestilla sibogae* (Miller and Hadfield, 1986), but is extremely variable in another opisthobranch, *Haminaea callidegenita* (Gibson, 1995). For planktotrophic larvae of the prosobranch genus *Crepidula,* the onset of competence is influenced by temperature, salinity, and nutritional history and is poorly predicted by size (Zimmerman and Pechenik, 1991; Pechenik *et al.,* 1996). Even after the onset of competence, responsiveness to an inducer can be affected by a larva's sensory history. Habituation to inducer has been studied in the gastropods *Phestilla sibogae* and *Haliotis rufescens* (Hadfield and Scheuer, 1985; Trapido-Rosenthal and Morse, 1986a, b). In the latter species, sensitization to an inducer by another external ligand has also been reported (Trapido-Rosenthal and Morse, 1985, 1986b), and both phenomenon have been related to regulation at the level of surface receptors for the inducer.

The structural and functional transformations that underlie the acquisition of competence and changes in responsiveness to inducers are not well understood. Competence may depend on the completion of neural networks that mediate between the initial chemosensory transduction of inducer and the behavioral and developmental events of metamorphosis. The modulation of such networks is an-

other possible cause of both age- and context-dependent shifts in responsiveness to inducers. Such networks are poorly characterized due to the difficulty of neurophysiological work in larvae (Arkett *et al.*, 1989; Barlow, 1990). In contrast, a wealth of information is available on the neural bases of many behaviors in adult gastropods, whose nervous systems are much more accessible. A common principle is that monoamine neurotransmitters and neurohormones often play critical roles in initiating and controlling behavior (Katz, 1995; Kabotyanski *et al.*, 1998). For example, dopamine is important in the control of several neural processes that are normally associated with a chemosensory stimulus: it has been shown to elicit feeding motor programs in several pulmonates (Quinlan *et al.*, 1997; Trimble and Barker, 1984; Wieland and Gelperin, 1983), modulate the activity of olfactory interneurons in *Limax maximus* (Gelperin *et al.*, 1993), and activate hunting behavior in the opisthobranch *Clione limacina* (Norekyan and Satterlie, 1993).

Given that gastropod veligers contain monoamines and other neuromodulatory transmitters (Kempf *et al.*, 1992, 1997; Pires *et al.*, 1997), it is reasonable to ask whether such compounds participate in the control of metamorphosis (Pires and Hadfield, 1991; Pires *et al.*, 1997; Couper and Leise, 1996; Froggett and Leise, 1999). We have used veligers of *P. sibogae*, which settle and metamorphose in response to an inducer derived from their adult prey, the coral *Porites compressa* (Hadfield, 1978; Hadfield and Pennington, 1990). The levels of catecholamines in these larvae increase several-fold within a period of 4–5 days after hatching, during which the larvae become competent for metamorphosis. α-methyl-DL-*m*-tyrosine (α-MMT), an inhibitor of the catecholamine-generative enzyme tyrosine hydroxylase (Uretsky *et al.*, 1975; Bernabe *et al.*, 1996), depletes catecholamines and inhibits induction of metamorphosis by coral inducer (Pires *et al.*, 1997). We now report that coral-induced metamorphosis is potentiated by exposure of competent larvae to L-DOPA, the synthetic precursor of catecholamines. This treatment substantially enhanced endogenous norepinephrine (NE) and especially dopamine (DA). L-DOPA treatment also restored catecholamine levels and ability to metamorphose in larvae that had been treated with α-MMT. Histochemical study of catecholamine-containing tissues suggested that enhancement of catecholamines was localized in populations of cells in locations consistent with those of cells exhibiting tyrosine hydroxylase-like immunoreactivity. Although these results support a role for catecholamines in the control of metamorphosis, experiments with younger larvae indicated that rising levels of catecholamines were not by themselves sufficient to account for the onset of competence.

Materials and Methods

Larval culture

Larvae were cultured as described by Miller and Hadfield (1986) with modifications given in Pires and Hadfield (1991). Larval ages are given as days postfertilization. All larvae were hatched at 5 d and held unfed in culture until needed. Veligers of *Phestilla sibogae* are facultative planktotrophs; under our culture conditions larvae begin to attain metamorphic competence at 7 d and by 10 d nearly all individuals are competent without having fed (Kempf and Hadfield, 1985; Miller and Hadfield, 1986).

Manipulation of catecholamine biosynthesis

Two sets of experiments were performed to test how manipulating catecholamine biosynthesis affected metamorphosis. In the first, content of endogenous catecholamines and frequency of metamorphosis were determined in 9-d larvae after treatment with α-MMT, L-DOPA (both from Sigma Chemical Co., St. Louis, MO), or both. (Endogenous catecholamines are depleted by α-MMT (Pires *et al.*, 1997), and preliminary experiments done for this study indicated that they are enhanced by L-DOPA at the concentrations used here.) Five replicates were performed on different culture batches on different dates. In each replicate, 1500–2000 larvae were taken from culture on day 8 and evenly divided among four treatments, all carried out in MBL artificial seawater (MBL-ASW; Cavanaugh, 1956) buffered to pH 8.2 with 10 m*M* Trizma: (1) 24 h in MBL-ASW alone; (2) 24 h in 1 m*M* α-MMT; (3) 21 h in MBL-ASW followed by 0.5 h in 10^{-5} *M* L-DOPA followed by 2 h in MBL-ASW; (4) 21 h in 1 m*M* α-MMT followed by 0.5 h in 10^{-5} *M* L-DOPA plus 1 m*M* α-MMT followed by 2 h in 1 m*M* α-MMT. L-DOPA was applied as a 0.5-h pulse followed by a MBL-ASW rinse because L-DOPA and other catechols oxidize rapidly in seawater and the oxidative products themselves affect metamorphosis (Pires and Hadfield, 1991). All treatments were carried out in 20-ml volume in 62-mm glass Stender dishes. At the end of the drug treatments, a sample of each group was homogenized for analysis of catecholamines by high-performance liquid chromatography (HPLC); the remainder was used to assay metamorphosis.

The second set of experiments tracked catecholamine content, frequency of metamorphosis, and onset of competence in larvae of increasing ages after modest or extreme enhancement of catecholamines by different doses of L-DOPA. Four replicate series were performed, beginning on different dates with different culture batches. In each replicate series about 1000 six-day-old larvae were pipetted from their culture into MBL-ASW, and then divided into three treatments, all in MBL-ASW: (1) 2–3 h in MBL-ASW alone; (2) 0.5 h in 5×10^{-7} *M* L-DOPA followed by 2 h in

MBL-ASW; (3) 0.5 h in 10^{-5} *M* L-DOPA followed by 2 h in MBL-ASW. A sample of each group was homogenized for analysis of catecholamines by HPLC; the remainder was used to assay metamorphosis. This experiment was repeated on days 7, 8, and 9 with larvae freshly drawn from the same culture in each replicate series. Nine-day-old larvae did not receive L-DOPA treatments; frequency of metamorphosis and catecholamine levels in these oldest larvae served for comparison with younger animals from the same batch that had been treated with L-DOPA on previous days.

Preparation of metamorphic inducer and assay of metamorphosis

Frequency of metamorphosis was assayed in response to preparations of the natural inducer. This "coral inducer" (CI) was made by incubating a few "fingers" of *Porites compressa* (about 50–75 g wet weight) in an 11.5-cm glass bowl filled with MBL-ASW. After 24 h the CI was filtered through coffee-filter paper, adjusted to 32 ppt salinity, and frozen in aliquots at −20°C. Aliquots to be used on a given day were thawed to room temperature and diluted with fresh MBL-ASW to make a graded dilution series of CI ranging up 0.5 times (0.5×) full strength (50% v/v CI in MBL-ASW). All experiments on a given culture batch of larvae used freshly thawed aliquots of the same CI. In each assay, 20–40 larvae were transferred to 37-mm glass Stender dishes containing 3 ml 0.05×, 0.1×, 0.2×, or 0.5× CI or MBL-ASW control. (For larvae that had been treated with α-MMT, the dilution series of CI used to assay metamorphosis also contained 1 m*M* α-MMT to maintain inhibition of catecholamine synthesis for the duration of the assay.) Assays were scored after 24 h. Individuals that had evacuated the larval shell were counted as having metamorphosed (Hadfield, 1978). Effects of CI concentration and of drug treatments on arcsine-transformed frequencies of metamorphosis were analyzed by ANOVA with Bonferroni adjustment for multiple comparisons (Zolman, 1993), using DataDesk 6.1 statistical analysis software (Data Description, Ithaca, NY).

Extraction of catecholamines and analysis by HPLC

For each determination of catecholamine content, 150–250 larvae were counted and pipetted into a glass homogenization tube on ice. The tube was briefly centrifuged (<150 × *g*), and excess water was drawn off to concentrate larvae in a volume of about 25 μl. Homogenization of larvae and extraction, separation, identification, and quantification of catecholamines by HPLC were modified from procedures described previously (Pires *et al.*, 1997). In summary, larvae were homogenized in a perchlorate buffer; supernatants of centrifuged homogenates were extracted over acid-washed alumina (Bioanalytical Systems) in 1.5 *M* Tris buffer (pH 8.6). Alumina was washed in deionized water and transferred to microcentrifuge filters, where excess water was spun off and catecholamines were eluted with 0.1 *M* perchloric acid. Efficiency of extraction of catecholamines, typically 60%–80%, was corrected with a dihydroxybenzylamine (DHBA) internal standard added to all larval homogenates. Catecholamines were separated on a Bioanalytical Systems MF-6213 analytical column (100 × 3.2 mm, 3 μm C-18 reversed phase packing). The aqueous portion of the mobile phase contained 100 m*M* monochloroacetic acid, 1.3 m*M* Na_2EDTA, and 1.3 m*M* sodium octyl sulfate. Mobile phase pH was adjusted between 3.0 and 3.3 with NaOH. The organic portion of the mobile phase was 2%–4% (v/v) acetonitrile. Flow rate was set at 1.0 ml/min. Catecholamines were detected with a Bioanalytical Systems LC-4C amperometric electrochemical detector with glassy carbon working electrode set at an oxidizing potential of 650 mV against a Ag/AgCl reference electrode. Detector output was low-pass filtered (1.0 Hz cutoff) and sent to a Hewlett-Packard 3395 integrator that printed chromatograms and calculated peak areas and retention times. Catecholamine contents were expressed as picomoles of free base per larva.

Histochemistry

At least 50 larvae were obtained from each group after scoring for frequency of metamorphosis in two replicates of the first set of experiments described above. In addition, several hundred 9-d larvae were taken directly from culture without drug treatment. All larvae were immobilized in a 1:1 mixture of MBL-ASW and 7.5% (w/v) $MgCl_2$ in water. These larvae were gathered into 1.5-ml microcentrifuge tubes and spun gently (<150 × *g* rpm) for 5–10 s and were then processed using the formaldehyde-glutaraldehyde (FaGlu) procedure modified from Furness *et al.* (1977) as follows: The MBL-ASW/$MgCl_2$ was replaced with FaGlu solution of 4% paraformaldehyde and 0.55% glutaraldehyde in phosphate-buffered saline (PBS: 50 m*M* Na_2HPO_4 − $7H_2O$ and 140 m*M* NaCl, pH 7.4). After 18 h, the fixed larvae were decalcified in 10% EDTA for 10 min, washed briefly in PBS, and then dehydrated through an ascending ethanol series (70%, 80%, 90%, 95%, 100%, 100%; 2 min/step). The larvae were then cleared and mounted on glass slides with methyl salicylate. The slides were viewed through a Zeiss Axiophot microscope equipped for ultraviolet (UV) epifluorescence with a filter block containing 365-nm excitation and 420-nm longpass barrier filters and photographed using Kodak T-MAX 400 film. Negative controls were also prepared with no glutaraldehyde added to the fixative. Such control preparations exhibited none of the blue-green fluorescence reported in this study.

Immunocytochemistry

Several hundred 9-d larvae taken directly from culture without any drug treatment were also processed for immunocytochemistry using monoclonal antibodies raised against tyrosine hydroxylase (TH; IncStar #22941, Stillwater, MN) and procedures similar to those employed previously in gastropods (Hernádi *et al.*, 1993; Voronezhskaya *et al.*, 1999; Croll *et al.*, 1999).

Larvae were first immobilized with $MgCl_2$ as described above and then fixed in 100% methanol at −20°C. (Preliminary experiments had indicated that such methanol fixation yielded more intense labeling than using paraformaldehyde in another gastropod, *Lymnaea stagnalis*, in which catecholaminergic neurons have been well characterized [Croll and Chiasson, 1990; Elekes *et al.*, 1991; Voronezhskaya *et al.*, 1999; Croll *et al.*, 1999].) After 2–4 h, the fixed larvae were decalcified in 10% EDTA and then placed in a blocking solution of PBS with the addition of 2% bovine serum albumin and 1% Triton X-100. This and subsequent steps were performed at 4°C. After 12 h, larvae were transferred to the primary anti-TH antibody diluted 1:50 in the blocking solution for 48 h. After three 30-min washes, larvae were incubated overnight in a 1:50 dilution of fluorescein isothiocyanate-labeled polyclonal goat anti-mouse antibodies (Sigma #F5262). Following several more washes in PBS, larvae were placed on glass slides and mounted in 3:1 glycerol in 0.1 *M* Tris buffer (pH 8.5) with the addition of 2% *n*-propyl gallate (Giloh and Sedat, 1982).

Preparations were examined either on the Zeiss Axiophot microscope equipped with a filter block containing 450–490 nm excitation and 515–565 nm bandpass barrier filters or on a Zeiss Axiovert microscope equipped for confocal laser scanning (Zeiss model LSM 510; excitation 488 nm with 505–550 nm barrier filter). In the latter case, photographs were produced by superpositioning stacks of 20–40 images obtained through stepped sequences of focal planes at about 1.0-μm intervals.

Negative control experiments used identical procedures except that the primary antibody was omitted. No labeling was observed in these control preparations.

Results

Depletion and enhancement of catecholamines in competent larvae

NE and DA were present in quantifiable amounts in larvae of all ages and all drug treatment groups used in this study. Figure 1 shows chromatograms of catecholamine standards and of alumina extracts of 9-d larvae that had been treated with MBL-ASW only, the tyrosine hydroxylase inhibitor α-MMT (1 m*M*), or the catecholamine precursor L-DOPA (10^{-5} *M*). Numbers of larvae and extraction efficiencies were very similar between treatments, so inspection of peaks roughly indicates relative amounts of catecholamines per larva. These chromatograms show the qualitative effects of catecholamine depletion by α-MMT and enhancement by L-DOPA, compared to larvae held in MBL-ASW. Enhancement of DA is especially evident in the chromatogram of L-DOPA-treated larvae (Fig. 1D). In addition, this chromatogram shows a prominent peak iden-

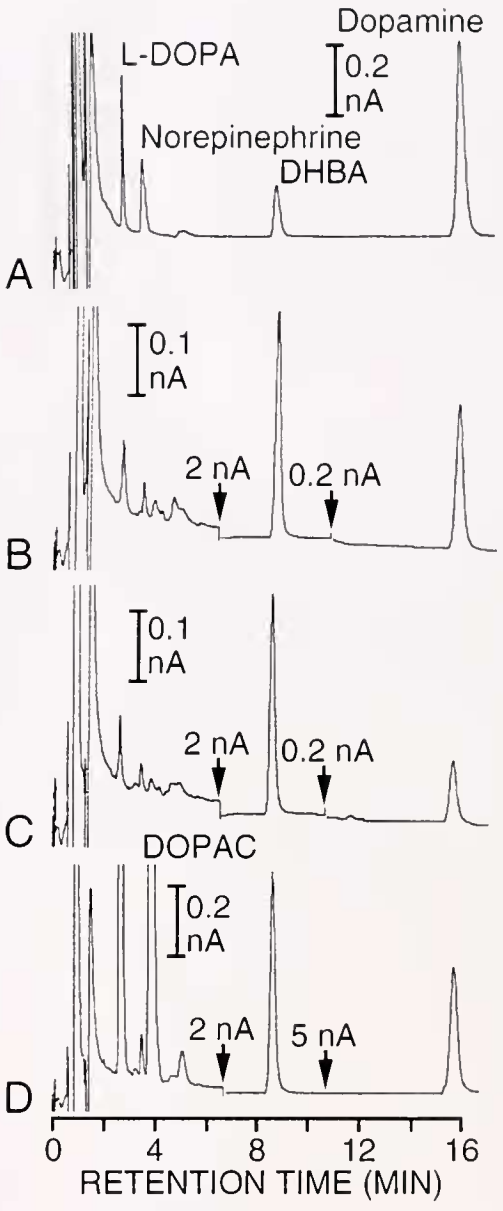

Figure 1. Chromatograms of catecholamine standards, and of alumina extracts of 9-d larvae of *Phestilla sibogae* from one replicate of an experiment in which catecholamine biosynthesis was manipulated. Standards (A) contained 0.32 pmol each of L-DOPA, norepinephrine, and dihydroxybenzylamine (DHBA), and 1.6 pmol of dopamine. (B) Extract of 210 larvae treated with MBL artificial seawater only; (C) extract of 210 larvae treated with 1 m*M* α-methyl-DL-*m*-tyrosine; (D) extract of 222 larvae treated with 10^{-5} *M* L-DOPA. Current scale of detector changes at points marked by vertical arrows.

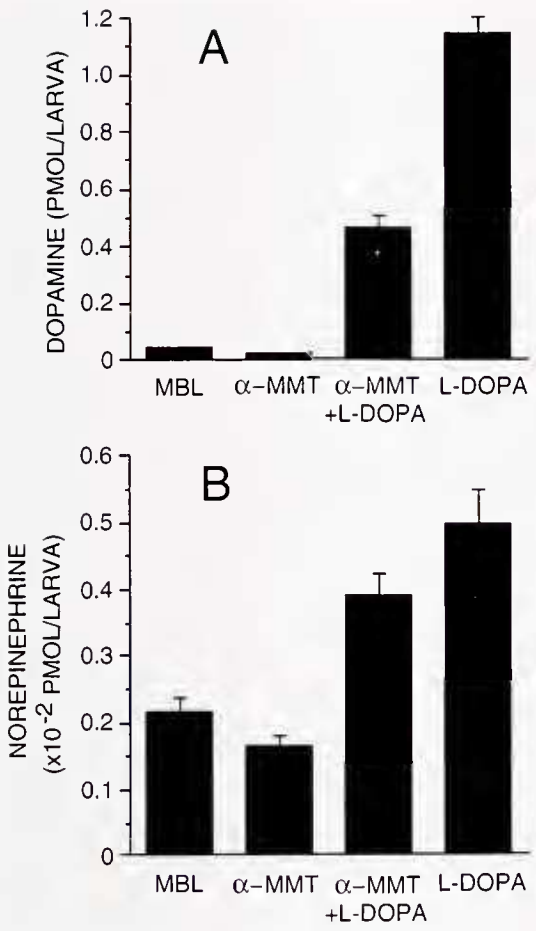

Figure 2. Content of dopamine (A) and norepinephrine (B) at 9 d postfertilization in larvae of *Phestilla sibogae* after treatment with 1 m*M* α-methyl-DL-*m*-tyrosine (α-MMT), 1 m*M* α-MMT plus 10^{-5} *M* L-DOPA, 10^{-5} *M* L-DOPA, or MBL artificial seawater control. Each column represents the mean (±1 SEM) of 5 replicate trials.

tified as the dopamine metabolite dihydroxyphenylacetic acid (DOPAC) on the basis of its co-elution with authentic DOPAC over a range of mobile phase pH and acetonitrile concentrations. Test extractions revealed that recovery of DOPAC was not accurately estimated by our DHBA internal standard, so we do not report quantitative values for DOPAC.

Treatment of 9-d larvae with a 0.5-h pulse of 10^{-5} *M* L-DOPA followed by a 2-h incubation in MBL-ASW resulted in an average 26-fold enhancement of endogenous DA (Fig. 2A), compared to larvae exposed to MBL-ASW alone. In contrast, DA was depleted to 49% of MBL-ASW control values in larvae that had been exposed to α-MMT (1 m*M* for 24 h). DA was elevated to 11 times the value of MBL-ASW controls in larvae that received a combined treatment of α-MMT and L-DOPA (1 m*M* α-MMT for 21 h, followed by an L-DOPA pulse as described above but in the continued presence of α-MMT). A similar pattern of depletion and enhancement of NE was observed following the above treatments (Fig. 2B), although the magnitude of these effects was much smaller than was observed for DA.

Analysis of variance revealed significant effects of culture batch ($P < 0.01$), CI concentration, drug treatment and (CI × drug) interaction (all $P < 0.0001$) on frequency of metamorphosis in 9-d larvae. No larva in any treatment metamorphosed in the absence of CI. Treatments with α-MMT and L-DOPA that modulated endogenous catecholamine levels had dramatic and opposite effects on the frequency of coral-induced metamorphosis (Fig. 3). Bonferroni *post-hoc* tests compared effects of individual drug treatments. Larvae that had been held 24 h in 1 m*M* α-MMT metamorphosed at much lower frequencies than control larvae held in MBL-ASW ($P < 0.0001$). Larvae that had received a 0.5-h pulse of 10^{-5} *M* L-DOPA, ending 2 h before exposure to CI, metamorphosed at significantly higher frequencies than did MBL-ASW controls when challenged with dilute preparations of CI ($P < 0.0001$). However, mean frequencies of metamorphosis were not significantly different in these two groups at the highest concentration of CI. Larvae that received a combined treatment of α-MMT and L-DOPA metamorphosed at frequencies that also were not significantly different than those of MBL-ASW controls.

Histochemistry

Nine-day larvae taken from two replicates of the experiment documented in Figures 2 and 3 were subjected to

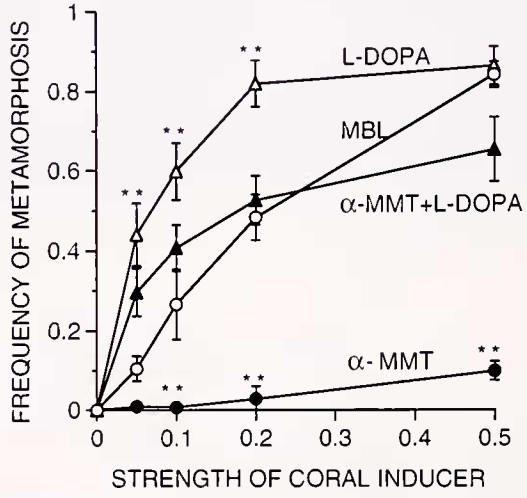

Figure 3. Frequency of metamorphosis of larvae of *Phestilla sibogae* after 24-h exposure to varying dilutions of coral inducer on day 9 postfertilization. Prior to inducer, larvae were subjected to the following treatments: 1 m*M* α-methyl-DL-*m*-tyrosine (α-MMT, filled circles), 1 m*M* α-MMT plus 10^{-5} *M* L-DOPA (filled triangles), 10^{-5} *M* L-DOPA (open triangles), or MBL artificial seawater control (open circles). Each point represents the mean (±1 SEM) of 5 replicate trials. Double asterisks indicate significant difference ($P < 0.01$) from MBL artificial seawater control at a given dilution of coral inducer.

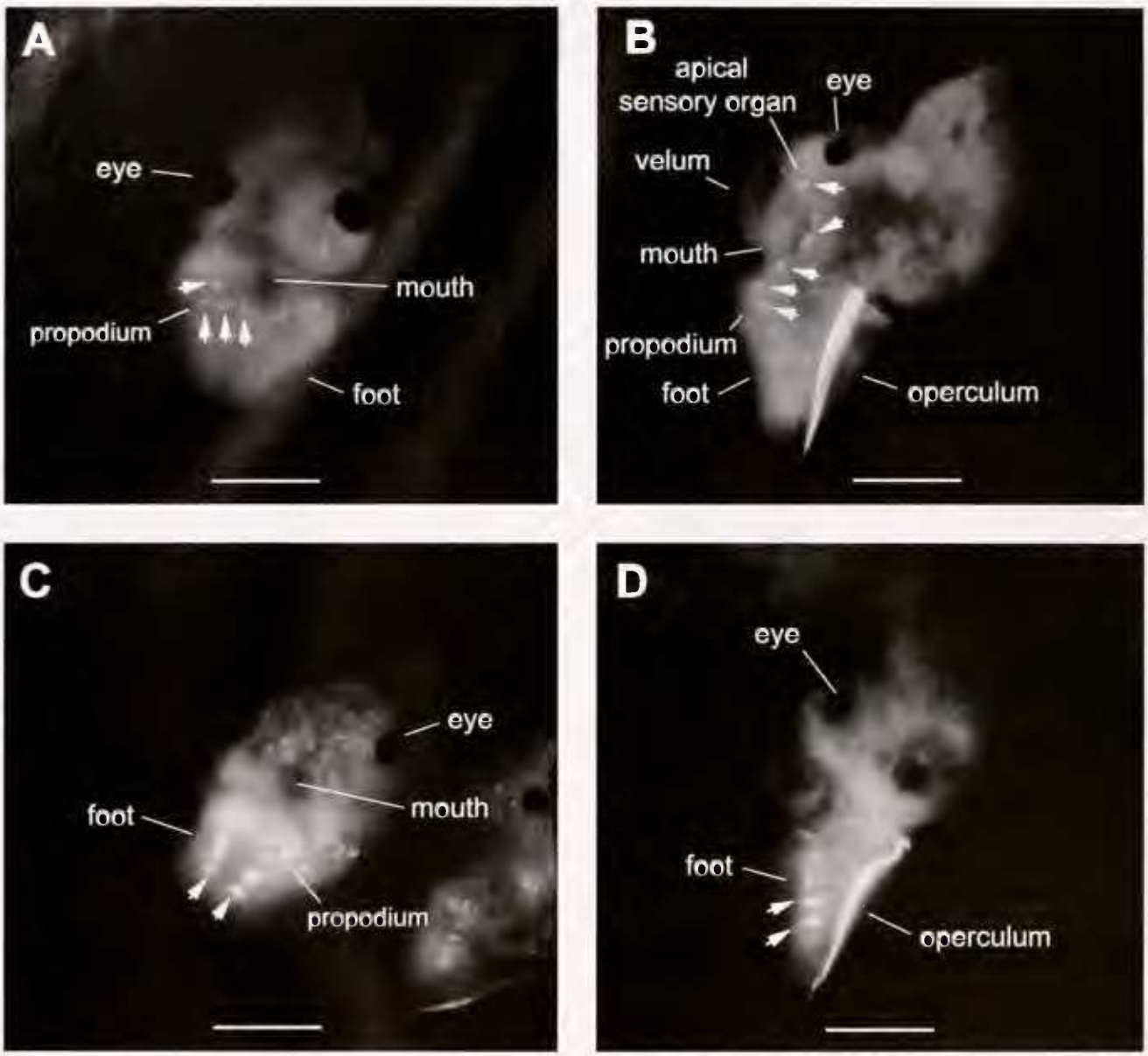

Figure 4. Aldehyde-induced histofluorescence. Frontal (A) and lateral (B) views of control larvae showing a few dimly fluorescent cells (arrowheads) in the region of the propodium, lateral to the mouth and near the apical sensory organ anterior to the eye. One or more cells were also sometimes observed near the base of the velum. No cells were generally found toward the distal tip of the foot to which the operculum is attached. Frontal (C) and lateral (D) views of larvae that were previously incubated in L-DOPA. Many more cells fluoresced brightly in such larvae. Most of these fluorescent cells were located in two rows running along the length of the foot (arrowheads in C) and in a patch on each side of the propodium. Several of these cells (arrowheads in D) appeared to be vase-shaped with slender processes projecting into the overlying epithelium. Calibration bars equal approximately 50 μm.

FaGlu histochemistry to visualize catecholamine-containing cells. The staining patterns in normal larvae without any prior drug treatment and those incubated in MBL-ASW or MBL-ASW plus α-MMT were all similar. Patches of 3 to 6 faint, blue-green fluorescent cells were reliably located in the propodial region of the foot of larvae from all of these groups (Fig. 4A). In addition, 2 to 4 fluorescent cells were located near the mouth, 1 to 2 cells were located more lateral to the mouth and near the base of the velum, and a final 2 to 4 fluorescent cells were located in regions anterior to the eyes (Fig. 4B).

The intensity of fluorescence and the numbers of fluorescent cells were markedly enhanced following treatment with L-DOPA alone or with L-DOPA plus α-MMT. In these

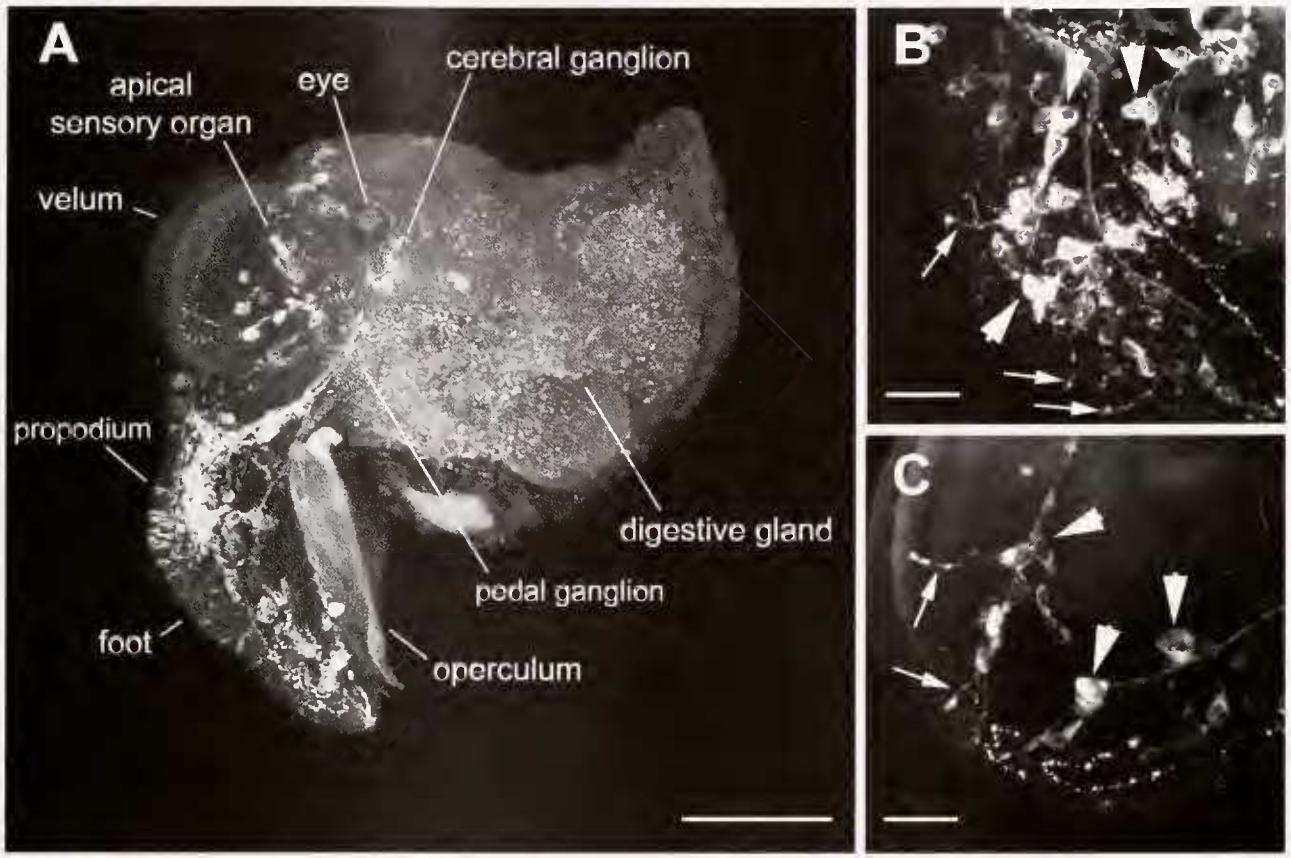

Figure 5. Tyrosine-hydroxylase-like immunoreactivity shown in projection patterns of normal larvae, reconstructed from multiple optic sections from a confocal microscope. (A) Lateral view of larva with velar lobes folded medially by mounting. A concentration of immunoreactive, subepithelial cells was labeled in the propodium. These cells appeared to send axons into the developing pedal ganglia. Large numbers of other cells were located along the length of the foot. Other cells were scattered near the mouth and velum, and in regions of the head near and dorsal to the apical sensory organ and eyes. (B) Magnified and more frontal view of somata (larger arrowheads) in propodium. Many of these cells possessed processes (slender arrows) that penetrated the overlying epithelium. (C) A smaller number of somata (arrowheads) with processes (slender arrows) are located near the distal tip of the foot. Calibration bar in A equals approximately 57 μm. Calibration bars in B and C equal approximate 12 μm.

larvae, the largest concentrations of fluorescent cells were again found in the propodial regions of the foot. Each of the bilateral patches contained 10 to 20 brightly fluorescent cells (Fig. 4C). In addition, several fluorescent cells were located in lateral bands along the length of the foot. At least some of these cells appeared to possess apical processes extending through the overlying epithelium (Fig. 4D).

Larvae treated with either L-DOPA or L-DOPA plus α-MMT also contained many brightly fluorescent cells in the head region. Several cells were routinely observed around the mouth, near the base of the velum (Fig. 4C), and anterior to the eyes. Thus the distribution of the cells in head regions was similar to that observed in normal larvae and in those incubated in MBL-ASW alone, or in MBL-ASW plus α-MMT. However, the cells observed in the head regions of larvae treated with L-DOPA or L-DOPA plus α-MMT appeared to be more intensely labeled and slightly more numerous.

Immunocytochemistry

Tyrosine-hydroxylase-like immunoreactive (TH-LIR) cells were labeled in 9-d larvae. The highest concentration of TH-LIR cells was located in bilateral patches in the propodium (Fig. 5A). Each patch contained about 40 to 50 cells (Fig. 5B). These cells appeared to be bipolar with one process penetrating the overlying epithelium and a long axon that projected to the region of the pedal ganglia. Similar cells were located at lesser densities on lateral edges along the length of the foot (Fig. 5C). Between 2 and 4 TH-LIR cells were located at each side near the mouth, and 2 to 4 additional cells were located more laterally near the

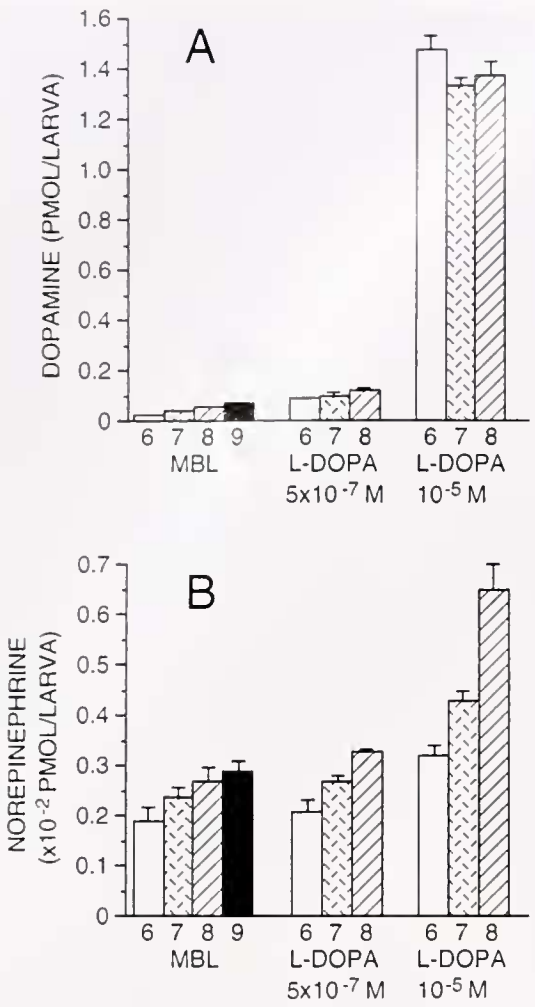

Figure 6. Content of dopamine (A) and norepinephrine (B) at 6, 7, 8, and 9 d postfertilization (numerals beneath horizontal axis) in larvae of *Phestilla sibogae*, following treatment with 5×10^{-7} *M* or 10^{-5} *M* L-DOPA, or MBL artificial seawater control. Each column represents the mean (±1 SEM) of 5 replicate trials for 10^{-5} *M* L-DOPA and MBL, or 4 trials for 5×10^{-7} *M* L-DOPA.

base of the velum on each side. At least one of these latter cells appeared to project an axon into the velum. An additional 4 to 6 somata were located anterior to the eyes, in regions near and also more dorsal to the apical sensory organ. Although TH-LIR fibers were contained in pathways running through the pedal and cerebral ganglia, no TH-LIR somata were located in these ganglia.

Enhancement of catecholamines in precompetent larvae

Treatment of 6-, 7-, and 8-d larvae with pulses of L-DOPA enhanced levels of catecholamines in a dose-dependent manner (Fig. 6). A 0.5-h pulse of 5×10^{-7} *M* L-DOPA raised dopamine levels by two- to threefold above control larvae of the same age held in MBL-ASW. Following a pulse of 10^{-5} *M* L-DOPA, dopamine was elevated to 22–50 times control levels, with the greatest degree of enhancement seen in the youngest larvae. Norepinephrine levels were slightly elevated above control values after a pulse of 5×10^{-7} *M* L-DOPA, and were only 2–3 times higher than controls after a pulse of 10^{-5} *M* L-DOPA.

The effects of L-DOPA on metamorphosis were analyzed by ANOVA in 6-, 7-, and 8-d larvae (Fig. 7). Metamorphosis was rare in 6-d larvae; treatment effects of Cl concentration were significant ($P < 0.005$), but effects of L-DOPA were not. Highly significant effects ($P \leq 0.0001$) of Cl concentration and of 10^{-5} *M* but not 5×10^{-7} *M* L-DOPA were obtained in 7-d and 8-d larvae. Treatment of 7-d larvae with 10^{-5} *M* L-DOPA was followed by low frequencies of metamorphosis in response to all nonzero concentrations of Cl; *post-hoc* comparisons revealed that these frequencies were significantly higher than in MBL-ASW control larvae at 0.1× Cl and 0.2× Cl ($P < 0.05$). At 8 d, larvae treated with 10^{-5} *M* L-DOPA metamorphosed at significantly higher frequencies than did control larvae upon exposure to 0.05× and 0.1× Cl ($P < 0.01$). As was the case with 7-d larvae, responses to the highest concentration of Cl (0.5×) did not differ significantly between 10^{-5} *M* L-DOPA-treated larvae and MBL-ASW controls. Only MBL-ASW control larvae were assayed for metamorphosis at 9 d. The response of these larvae to Cl was similar to that of 9-d MBL-ASW control larvae in the first series of experiments.

Discussion

Pharmacological manipulations of catecholamine biosynthesis had profound effects on metamorphosis in response to Cl in 9-d competent larvae of *Phestilla sibogae* (Fig. 3). Treatment of larvae with a 0.5-h pulse of 10^{-5} *M* L-DOPA enhanced contents of NE (more than twofold) and especially DA (26-fold), compared to control levels (Fig. 2). This treatment potentiated metamorphic responses to all but the highest concentration of Cl, compared to MBL-ASW controls. At the highest concentration of Cl, the mean frequencies of metamorphosis of control and L-DOPA-treated larvae were statistically indistinguishable (0.84 and 0.86, respectively) and may reflect metamorphosis of all larvae that were competent at that age (Miller and Hadfield, 1986). The catecholamine-depleting drug α-MMT inhibited metamorphosis over a range of Cl concentrations, compared to controls treated only with MBL-ASW before exposure to Cl (Pires *et al.*, 1997). In larvae that had been treated with 1 m*M* α-MMT for 24 h, DA was depleted to 48% of the control value measured in MBL-ASW-treated larvae, whereas NE was depleted only to 76% of the control value. It is likely that inhibition by α-MMT was due to depletion of catecholamines and not to an unknown toxic effect of α-MMT, because larvae treated with α-MMT and then pulsed with L-DOPA metamorphosed at high frequencies in

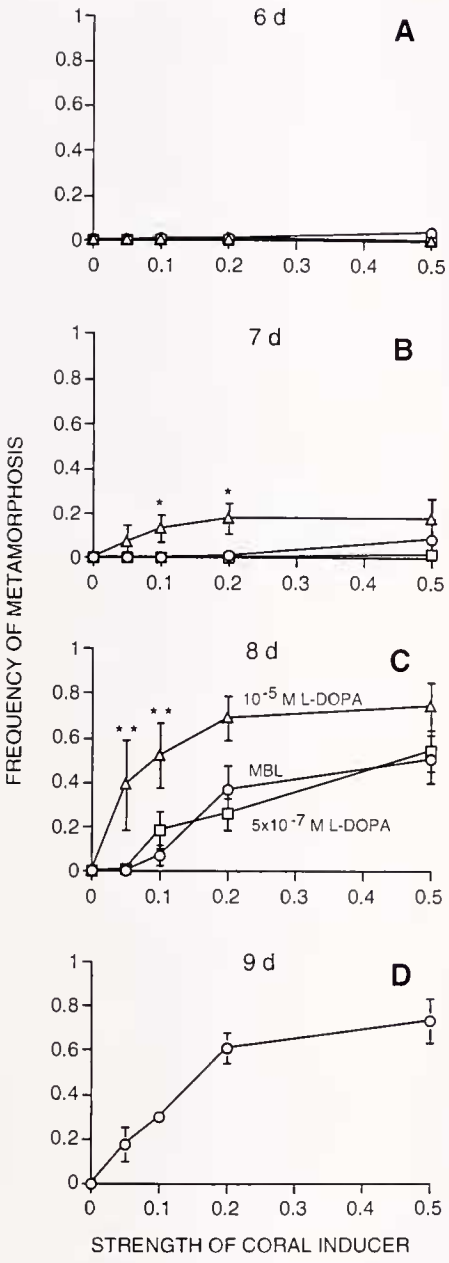

Figure 7. Frequency of metamorphosis of larvae of *Phestilla sibogae* after 24-h exposure to varying dilutions of coral inducer, following 0.5-h treatment with 5×10^{-7} *M* (squares) or 10^{-5} *M* (triangles) L-DOPA, or MBL-artificial seawater control (circles) on day 6 (A), 7 (B), 8 (C), or 9 (D, control only) postfertilization. Each point represents the mean (±1 SEM) of 5 replicate trials of 10^{-5} *M* L-DOPA and MBL, or 4 trials for 5×10^{-7} *M* L-DOPA. Asterisks indicate significant difference (*, $P < 0.05$; **, $P < 0.01$) from MBL artificial seawater control at a given dilution of coral inducer.

response to CI even in the continued presence of α-MMT. Larvae that received this combined treatment of L-DOPA and α-MMT had levels of DA and NE that were higher than those of MBL-ASW controls but lower than those measured in larvae treated with L-DOPA only. The mechanism by which α-MMT depletes endogenous DA, at least in mammalian nervous systems, involves both inhibition of tyrosine hydroxylase activity by α-MMT and displacement of stored DA by a decarboxylated metabolite of α-MMT (Uretsky *et al.*, 1975; Bernabe *et al.*, 1996). The latter mechanism may help explain why larvae treated with both α-MMT and L-DOPA contained less DA and NE than those treated with L-DOPA alone. Taken together, our manipulations of catecholamine biosynthesis with L-DOPA and α-MMT in competent larvae show that depletion of endogenous catecholamines is accompanied by inhibition of CI-induced metamorphosis, whereas enhancement of catecholamines is accompanied by potentiation of metamorphic responses to CI. Thus catecholamines appear to serve as endogenous modulators of metamorphosis in competent larvae of *P. sibogae*.

Catecholamine-containing cells in competent larvae were labeled by FaGlu histochemistry (Fig. 4) in order to map locations of these putative modulators of metamorphosis, and to test the anatomical specificity of catecholamine enhancement by L-DOPA. Intensity of labeling and numbers of labeled cells were dramatically enhanced by treatment of larvae with L-DOPA, without a concomitant increase in background fluorescence. Thus, the quantitative enhancement of catecholamine levels measured by HPLC in such larvae was localized in specific populations of cells. The locations of these cells were consistent with the locations of cells labeled with antibodies to tyrosine hydroxylase (Fig. 5). Immunocytochemistry combined with confocal microscopy, however, revealed larger numbers of cells than did FaGlu histochemistry. The former method presumably labeled a catecholamine-generative enzyme rather than catecholamines themselves, and yielded preparations with lower background fluorescence. Unfortunately, differences in fixative solutions for FaGlu and immunocytochemistry prevented double-labeling of cells in this study. Nonetheless, it remains likely that the cells labeled with FaGlu whose catecholamine contents were increased by L-DOPA treatment were cells that are capable of synthesizing catecholamines, but normally contain concentrations below the level of detection by histochemistry.

The location and morphology of TH-LIR cells hint at mechanisms by which catecholamines might modulate metamorphosis. Several of these cells are located near, and perhaps within, the apical sensory organ, which has been implicated as the chemosensory organ responsible for transduction of the CI stimulus (Kempf *et al.*, 1997; Hadfield *et al.*, 2000). Future studies must further examine the exact locations and morphologies of these cells, since they either may be chemosensory cells themselves or may modulate synaptic interactions between primary chemosensory neurons and postsynaptic cells in a signaling pathway. Dopamine is known to modulate the activity of procerebral olfactory interneurons in the pulmonate mollusc *Limax*

maximus (Gelperin *et al.*, 1993) as well as several aspects of central olfactory processing in amphibians and mammals (Duchamp-Viret *et al.*, 1997; Doty *et al.*, 1998). One or two pairs of TH-LIR cells were located at the base of the velum, and at least one pair appeared to send an axon distally into the ipsilateral velar lobe. Dopamine inhibits ciliary beating in gill epithelium of the bivalve *Mytilus edulis* (Catapane *et al.*, 1978; Aiello *et al.*, 1986) and also inhibits velar ciliary beating in the larvae of this species (Beiras and Widdows, 1995a). Dopaminergic innervation of the velum of *P. sibogae* might possibly serve to inhibit ciliary beating as part of the settlement response to CI that precedes the initiation of metamorphosis (Hadfield, 1978). Many cells exhibiting TH-LIR and aldehyde-induced histofluorescence were present in the propodium of the foot. These cells possessed apical processes extending to the surface, a characteristic of sensory neurons; in immunocytochemical preparations some of these same cells were also seen to have axonal projections to the pedal ganglia. Similar cells have been labeled by TH-LIR in anterior regions of the foot in embryonic and postembryonic stages of the pulmonate *Lymnaea stagnalis*, and by aldehyde-induced histofluorescene in ventral anterior regions of the foot in pediveligers of the bivalves *Mytilus edulis* and *Placopecten magellanicus* (Croll *et al.*, 1997; Voronezhskaya *et al.*, 1999; Croll *et al.*, 1999). In *P. sibogae* the propodium becomes enlarged around the time of metamorphic competence, mostly due to the development of mucus-secreting glands (Bonar and Hadfield, 1974). Settlement in response to CI is accompanied by secretion of mucus from the foot, and normally there is extensive contact between the propodium and the substratum. Therefore, potentiation of a sensory or motor function of the foot associated with a settlement response to CI is a means by which catecholaminergic cells might promote the initiation of metamorphosis. Sensory neurons in the anterolateral propodial ganglia of veligers of the nudibranch *Onchidoris bilamellata* are depolarized by a soluble settlement cue that is derived from barnacle prey of adult *O. bilamellata* (Chia and Koss, 1989; Arkett *et al.*, 1989). Neither such cells nor such ganglia have been described in veligers of *P. sibogae*, but we cannot rule out the possibility that homologous cells in this species might have a role in chemosensory responses to CI.

Our final set of experiments investigated the relationship between levels of endogenous catecholamines and the onset of metamorphic competence in *P. sibogae*. Under our culture conditions, larvae normally begin to attain competence on day 7; most are competent on day 8, and nearly all are competent on day 10 (Miller and Hadfield, 1986). Previous work had indicated that larval contents of DA and NE increase severalfold during the period of 4 or 5 days between hatching and attainment of maximal competence (Pires *et al.*, 1997). In larvae of the bivalve *Crassostrea gigas*, there is a sharp increase in NE content that coincides with the onset of competence (Coon and Bonar, 1986); in that species, dopamine and norepinephrine have been further implicated in the control, respectively, of settlement and metamorphosis (Coon and Bonar, 1987; Bonar *et al.*, 1990; Beiras and Widdows, 1995b). These observations, together with the correlation of catecholamine enhancement and potentiation of metamorphosis in 9-d larvae in the present study, led us to ask whether the timing of competence in larvae of *P. sibogae* could be influenced by pharmacological enhancement of catecholamines. Larvae aged 6, 7, and 8 d were treated with either a low (5×10^{-7} *M*) or high (10^{-5} *M*) dose of L-DOPA, or with MBL-ASW, then assayed for catecholamine contents (Fig. 6) and for metamorphic response to CI (Fig. 7) as in the first set of experiments. The higher dose was the same as that used in the first set of experiments on 9-d competent larvae and also yielded dramatic increases in contents of catecholamines, especially DA, in younger larvae. Dopamine increased to a similar high level, 1.3–1.5 pmol/larva, in 6-, 7-, and 8-d larvae. These levels greatly exceed the range of values normally found in larvae and juveniles of *P. sibogae* (Pires *et al.*, 1997), and indicate that the ability to convert L-DOPA to DA and store it is already well developed by 6 d. The lower dose of L-DOPA brought about much more modest enhancements of DA and NE within their normal physiological ranges, in most cases close to levels typically found in older (9-d) competent larvae. Frequency of metamorphosis was always very low in 6-d larvae, never exceeding 0.1 even at the highest concentration of CI, and was not significantly affected by either dose of L-DOPA. In 7-d and 8-d larvae the higher dose but not the lower dose of L-DOPA significantly potentiated metamorphosis in response to CI, compared to controls treated with MBL-ASW.

The results of these final experiments clearly indicate that the increases in catecholamine levels that naturally occur around the time of competence, mimicked by exposure to the lower dose of L-DOPA, are not sufficient to confer competence. *Post-hoc* analysis of metamorphosis at each concentration of CI in 7-d and 8-d larvae revealed that the higher dose of L-DOPA significantly increased frequencies of metamorphosis at low concentrations of CI, but not at the highest concentration. After this L-DOPA treatment, the responses of larvae to CI saturated at frequencies of metamorphosis that were not significantly different from the responses of MBL-ASW control larvae to the highest concentration of CI. This suggests that potentiation of metamorphosis by L-DOPA operated mostly in larvae that had already become competent. However, we cannot exclude the possibility that the apparent differences between 10^{-5} *M* L-DOPA and MBL-ASW control treatments at the highest concentration of CI in Figure 7B and C reflect a small positive effect of catecholamine enhancement on the onset of competence.

How might catecholamines modulate metamorphosis in

competent larvae of *P. sibogae* in nature? Both DA and NE are very weak inducers of metamorphosis when bath-applied in the absence of CI (Pires and Hadfield, 1991). Therefore it seems unlikely that either of these serves as the major neurotransmitter in a signaling pathway that directly mediates the induction of metamorphosis. The inhibition of coral-induced metamorphosis in larvae depleted of catecholamines by α-MMT (Fig. 3) suggests that catecholaminergic cells may modulate metamorphosis in the sense of gating or enabling (Harris-Warrick, 1988) a primary signaling pathway. This sort of modulatory function has been demonstrated in the serotonergic cerebral giant cells of the pulmonate *Lymnaea stagnalis*, which enable activation of a feeding motor program by other interneurons (Yeoman *et al.*, 1994). Pharmacological depletion of serotonin in this species lowers the proportion of animals that respond to a food stimulus, and increases the latency and diminishes the intensity of responses that do occur (Kemenes *et al.*, 1990). In similar fashion, depletion of catecholamines in modulatory neurons in larvae of *P. sibogae* may lower the proportion of animals that respond to CI.

Potentiation of coral-induced metamorphosis of L-DOPA was observed only after enhancement of catecholamine contents, especially DA, far beyond normal physiological values. Although we have demonstrated that these substances load into specific populations of cells, we remain ignorant of the extent of physiological release of catecholamines in these experiments and of the conditions that favor catecholamine release generally. Indeed, there is evidence in other systems that stored catecholamines are segregated into releasable and non-releasable pools (Kelly, 1993; Reimer *et al.*, 1998). Enhancement of release in L-DOPA-treated larvae would depend both on the presence of some of the elevated catecholamine contents in releasable cellular pools and on the level of activity in those cells. The enhancement of catecholamine release in our experiments may have been much more modest than the enhancement of total catecholamine contents, especially if we did not provide adequate stimuli to evoke activity in the appropriate cells. Under natural conditions, such catecholaminergic modulatory cells, carrying a normal complement of transmitter, might potentiate responses to CI by increasing their activity in the context of sensory cues or behaviors that precede or accompany natural settlement. The activity of serotonergic modulatory cerebral giant cells of adult molluscs has been shown by *in vivo* recording to be strikingly dependent on behavioral context—for example, whether the animal is quiescent, locomoting, or feeding, and whether or not a food stimulus is present (Weiss *et al.*, 1978; Yeoman *et al.*, 1994). Most assays of metamorphic responses to natural inducers in *P. sibogae* and other marine invertebrates have been performed with filtered extracts of inducers in laboratory glassware under static conditions. Intriguingly, responses to CI in larvae of *P. sibogae* are potentiated by mechanical agitation of the assay dishes (Pechenik *et al.*, 1995). More assays that better incorporate the sensory richness of a larva's experience leading up to natural metamorphosis might uncover behavioral contexts that allow modulation of responses to inducers.

Acknowledgments

This work was supported by Dickinson College, Natural Sciences and Engineering Research Council of Canada grant OPG 38863, and U.S. Office of Naval Research grant N00014-95-1-1015.

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